Synergy Between Irradiation and Chlorination in Killing of *Salmonella, Escherichia Coli* O157:H7, and *Listeria Monocytogenes*

DONALD W. THAYER, GLENN BOYD, AND WILLIAM F. FETT

ABSTRACT: The objectives of the studies were to determine if radiation and chlorine acted in a simple additive process or if a nonadditive increase in inactivation occurred when chlorination followed γ-irradiation both in vitro and in situ. Separate studies evaluated the effects of γ-irradiation (0.2 kGy at 20 °C), chlorination (0.5 ppm for 10 min), or irradiation followed by chlorination of *Salmonella*, *Escherichia coli* O157:H7 Ent C9490, or *Listeria monocytogenes* cells suspended on membrane filters. Cocktails of *Salmonella enterica* serovars: Anatum, Infantis, Newport, and Stanley or of *L. monocytogenes* isolates: ATCC 7644, 15313, 43256, and 49594 (Scott A) were used. In each case, greater inactivation was observed from irradiation followed by chlorination than was predicted from the sum of the inactivation of the 2 treatments when applied separately. Inactivation of *Salmonella* cells was determined also with the pathogens adsorbed onto alfalfa seeds. Analysis of results of 3 sets of experiments with *Salmonella* adsorbed onto alfalfa seeds also led to the conclusion that the combination treatments were synergistic and produced a greater inactivation than were expected from the sums of the treatments although experiments with larger number of seeds gave less evidence of synergy. The effectiveness of both interventions against Salmonella was significantly reduced when the pathogen was on alfalfa seeds.

Keywords: chlorine, *E. coli*, γ-radiation, *L. monocytogenes*, Salmonella

Introduction

The microbiological safety of sprouted seeds has remained questionable even though pretreatment of the seed before sprouting with calcium hypochlorite at concentrations of 20000 μg/mL is often practiced by growers in the U.S.A. (Thomas and others 2003). For example, in 1999 there were 2 outbreaks of salmonellosis due to the ingestion of alfalfa and clover sprouts that were grown from seeds treated with calcium hypochlorite (Brooks and others 2001; Proctor and others 2001). Since 1996 there have been 27 outbreaks of foodborne illness associated with contaminated sprouts with over 1500 cases of illness (Food and Drug Administration 2004). Due to the continuing sprout-related outbreaks of foodborne illness, including 2 in 2004, in May of 2005 the FDA held a public meeting to solicit comments on the current science related to foodborne illness associated with the consumption of sprouts (Food and Drug Administration 2005).

The authors demonstrated previously that treating alfalfa and broccoli seeds with approximately 2 kGy of γ-radiation inactivated 2 to 3 logs of contaminating *Salmonella* cells and that high quality sprouts could be grown from the irradiated seeds (Thayer and others 2003a; Thayer and others 2003b; Rajkowski and others 2003). Unfortunately, these same studies indicated that sprout yield decreased when alfalfa sprouts were irradiated to doses greater than 2 kGy. These results led to the conclusion that irradiated seeds should be treated by the grower also with calcium hypochlorite. The question remained, however, whether such combination treatments would produce additive or synergistic effects. The objective of this study was to determine if the inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes,* or *Salmonella* cells by γ-irradiation followed by chlorination is additive or synergistic (greater than the sum of the effects of individual treatments).

Materials and Methods

Experimental design and concept

Our concept was that synergistic results might occur when different antimicrobial agents are used that have different mechanisms of action. The 1st treatment inactivates some cells but only injures others. The 2nd treatment acts upon an injured population and thus produces a greater-than-expected inactivation of the microorganisms. To test this concept we proposed to use doses of radiation and calcium hypochlorite that would produce a clearly measurable effect but which were sufficiently low that when both treatments were applied the combined results would be clearly demonstrable. For example, using a bacterial culture of approximately 10^6 cells per mL or gram we aimed for a dose that would achieve about a 2 log inactivation with each agent. The effect of each treatment and/or the sequential treatment would thus be easily quantified. Initial studies were conducted in the absence of alfalfa seeds since the seeds also react with both radiation and chlorine. If synergy occurred between the antimicrobial agents or treatments in the absence of the seeds then the theory would be tested using inoculated seeds first, in as nearly as possible the same conditions under which synergy had been demonstrated, then under conditions suitable for testing of industrial processes. These concepts led to the following experimental designs.

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Cultures

The following *Salmonella enterica* serovars have been associated with outbreaks of salmonellosis after ingestion of sprouts: *Salmonella* Anatum F4317, *Salmonella* Infantis F4319, *Salmonella* Newport H1275, and *Salmonella* Stanley H0558 and were obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., U.S.A. *Escherichia coli* O157:H7 Ent C9490 was associated with a ground-beef-related outbreak of disease and was obtained from the Oregon State Public Health Laboratory, Portland, Ore., U.S.A. *Listeria monocytogenes* ATCC 7644, 15313, 43256, and 49594 (Scott A) were obtained from the American Type Culture Collection (Manassas, Va., U.S.A.). Each isolate was maintained and cloned on tryptic soy agar (TSA, Difco/BBL, Sparks, Md., U.S.A.). Culture identity was confirmed by Gram stains and from reactions on the Gram-Negative or Gram-Positive Identification Cards, as appropriate, of the Vitek AMS Automicrobic System (bioMérieux Vitek, Inc. U.S.A., Hazelwood, Mo., U.S.A.) (Aldridge and others 1977; Knight and others 1990). Each isolate was cultured independently in 100 mL of tryptic soy broth (TSB, BBL/Difco) at 37 °C for 18 h. Equal amounts of the individual isolates of *Salmonella,* or *L. monocytogenes,* as appropriate, were mixed just prior to use to prepare an inoculum cocktail. A 3rd inoculum consisted of the single strain of *E. coli* O157:H7 cultured in TSB, as described above.

Irradiation

The self-contained, γ-radiation source (Lockheed Georgia Co., Marietta, Ga., U.S.A.) has 23 ^{137}Cs pencils placed in an annular array around a 63.5 cm high stainless steel cylindrical chamber with a 22.9-cm internal dia. The source strength at the time of the study was approximately 103841 Ci (3.84 PBq) (The becquerel (Bq) is the SI unit of radioactivity. 1 Bq = 1 nuclear transformation per second; PBq: petabecquerels = 10^{15} Bq) with a dose rate of 0.10 kGy min^{-1}. The dose rate was established using alanine transfer dosimeters from the Natl. Inst. of Standards and Technology, Gaithersburg, Md., U.S.A. Corrections for source decay were made monthly. Routine dosimetry was performed using 5-mm-dia alanine dosimeters (Bruker Biospin Corp., Billerica, Mass., U.S.A.), and the free-radical signal was measured using a Bruker EMS 104 EPR Analyzer (ISO/ASTM 2002). Variations in sample dose absorption were minimized by placing small samples within a uniform area of the radiation field, by irradiating the samples within a polypropylene container (4-mm wall) to absorb Compton electrons, and by using the same geometry for sample irradiation during each study. Under these conditions the target dose and the actual absorbed dose were the same within the limits of dosimetric measurement. Based on measurement of dosimeter responses in several experiments, actual doses were within 2% of target. Samples were maintained at 20 ± 1 °C during irradiation by the thermocouple-controlled injection of the gas phase from liquid nitrogen into the top of the irradiation chamber. Sample temperature was monitored continuously with thermocouples that were taped to 2 samples within the chamber.

Studies of antimicrobial treatments of pathogen cells in vitro on membrane filters

For control samples bacterial inocula were diluted in sterile Butterfield's phosphate (BP) (0.25 M KH_2PO_4 adjusted to pH 7.2 with NaOH) to approximately 10^6 CFU/mL, and 1 mL of the diluted inoculum was added to a tube containing 19 mL of sterile BP. After mixing, cells were collected by filtration on sterile cellulose nitrate, 0.45 μm, 47-mm filters (Millipore, Bedford, Mass., U.S.A.). Filters were removed aseptically and placed in a Stomacher Lab System No. 400 bag with side filter. After addition of 99 mL of BP, the bag was placed in a Stomacher lab blender (model 400, Tekmar Co., Cincinnati, Ohio, U.S.A.) and pummeled for 90 s. The suspensions were then assayed for colony-forming units (CFU) using standard pour-plate methods with serial dilutions prepared in sterile BP. Pour plates were prepared with TSA and incubated at 37 °C for 24 h. CFU were counted with a Biologics Accucount 1000 automated colony counter at a dilution giving 30 to 300 CFU per plate.

Gamma-irradiated samples

For the γ-irradiated samples the inoculum was diluted to approximately 10^6 CFU/mL in sterile BP, and 1 mL of this culture solution was placed into a tube containing 19 mL sterile BP. After mixing the tube was irradiated to 0.2 kGy at 20 °C. Following irradiation, tube contents were filtered. Filters were placed in a filter Stomacher bag with 99 mL of BP and pummeled for 90 s. Suspensions were assayed for survivors.

Cl_2-treated samples

For the Cl_2-treated samples the inoculum was diluted in sterile BP to approximately 10^6 CFU/mL, and 1 mL was added to 19 mL of sterile BP. The contents of this tube were filtered and the filter left on the apparatus. Next, 20 mL of 0.5 ppm Cl_2 solution was added to the filter apparatus and allowed to stand for 10 min. The 0.5 ppm Cl_2 solution was prepared by dilution of a 3% w/v solution of calcium hypochlorite (Sigma-Aldrich, St. Louis, Mo., U.S.A.) with 65% available chlorine (20000 ppm) with sterile deionized water. The chlorine concentration was measured with the Reflectoquant system Model 16950 and Reflectoquant test strips (EM Science, Gibbstown, N.J., U.S.A.). The Cl_2 solution was removed by filtration, and the filter was rinsed with 20 mL sterile BP and placed in a Stomacher bag. The filter was pummeled in the bag with 99 mL of BP and assayed for surviving CFU, as were the control and γ-irradiated samples.

Gamma-irradiated then Cl_2-treated samples

The inoculum was diluted to approximately 10^6 CFU/mL in sterile BP; 1 mL was added to 19 mL sterile BP, and the tubes were irradiated, as were the γ-irradiated samples, as stated above. The irradiated samples were then treated with calcium hypochlorite solution in the same manner as were the samples treated only with Cl_2, as stated above. The filter was placed in a Stomacher bag, pummeled, and the suspension was assayed for surviving CFU, as stated above.

Three independent experiments on inactivation of *Salmonella* were conducted with 3 replicates per study and 3 Petri plates counted per replicate. Three independent experiments with 1 replicate per treatment, each with 3 Petri plates counted per replicate, were conducted on inactivation of *E. coli* O157:H7 and *L. monocytogenes*.

Studies of antimicrobial treatments of *Salmonella* cells on 0.1 g of alfalfa seeds on membrane filters

Equal amounts of overnight cultures of each serovar were placed in a sterile bottle and mixed. The seeds (50 g) were placed in the inner filter of a Stomacher Lab System No. 400 filter bag. The cell mixture (50 mL) was then added to the outer portion of the bag and the bag heat sealed. The bag was hand massaged for 60 s to wet the seeds. Next, the top of the bag was cut open and all excess liquid was squeezed out of the bag into a waste container. The seeds were then placed aseptically into sterile Petri dish halves in a monolayer, and these dishes were placed inside a vacuum desiccator containing fresh desiccant (Drierite indicating 97% $CaSO_4$, 3% $CaCl_2$ [WA Hammond Drierite Co. Ltd, Xenia, Ohio, U.S.A.]) and the seeds allowed to dry overnight *in vacuo*. After the vacuum was released from the desiccator the Petri dish halves were removed, and 0.1 g samples of the seeds (about 40 to 50 seeds) were weighed out. Samples

were designated as "control" (no γ, no Cl_2), "γ-irradiated" (0.2 kGy), "Cl_2-treated" (0.5 ppm), and "γ-irradiated then Cl_2-treated."

Control seed samples

The control samples were first ground in a sterile handheld stainless steel grinder (Glas-Col, Terre Haute, Ind., U.S.A.) so that after 10 to 15 "back-and-forth" motions the seeds were reduced to a powder. Using 1 mL portions of sterile BP, the grinder was rinsed 3 times; the rinsate was recovered in a pipette each time, and 3 mL was added to a tube containing 17 mL sterile BP. After mixing, the contents of the tube were filtered and assayed for CFU as described above.

Gamma-irradiated seed samples

The samples designated as "γ-irradiated" were placed in filter-Stomacher bags, which were then heat sealed and irradiated to 0.2 kGy at 20 °C. Following irradiation the seeds were ground and assayed for surviving CFU, as described above.

Cl_2-treated seed samples

The seeds for the Cl_2-treated samples were placed on top of a sterile cellulose nitrate, 0.45 μm, 47-mm filter; then, 40 mL of 0.5 ppm Cl_2 solution was added and allowed to stand for 10 min. The Cl_2 solution was then removed by filtration, and the seeds were rinsed on the filter with 40 mL of sterile BP. The available Cl_2 concentration was measured, as described above. The seeds were then ground and assayed for surviving CFU, as described above.

Gamma-irradiated then Cl_2-treated seed samples

The seeds designated "γ-irradiated then Cl_2-treated" were irradiated as described above for the γ-irradiated samples, and then treated with Cl_2 as described above for the Cl_2-treated samples. The seeds were then ground and assayed for CFU, as described above. It should be noted that the length of time needed to prepare the samples required that they be held overnight at 2 °C before assay.

Studies of antimicrobial treatments of *Salmonella* cells on 5.0 g of alfalfa seeds on membrane filters

The procedures followed those for 0.1 g of seeds but with a sample size of 5.0 g of unground alfalfa seeds. A chlorine treatment was performed with a calcium hypochlorite solution containing 5000 ppm Cl_2. At 10 min, the first 10-fold dilution was made with D/E Neutralizing Broth (Difco/BBL). The broth was added to neutralize residual chlorine adsorbed by the seeds, limiting the exposure time to 10 min. The larger mass of seeds and the addition of the neutralizing step were determined by preliminary experimentation to require a chlorine concentration of 5000 ppm and a γ-radiation dose of 2.0 kGy to achieve the desired effects on the population of *Salmonella* on the seed.

Determination of the *D*-value for the inactivation of *Salmonella* on 10 g of seed by γ-irradiation and inactivation or by γ-irradiation followed by chlorination

This study was designed to replicate potential industrial practices for decontamination of food-sprout seeds on a laboratory scale. If synergy occurs between irradiation and chlorination, the irradiation *D*-value for inactivation of *Salmonella* should decrease when irradiated seeds are treated with chlorine. Inoculated seeds were weighed out in 10 g amounts and placed into the sample compartment of filter Stomacher bags, which were then heat sealed. Six independent experiments were conducted. Each study included a nontreated control, a nonirradiated sample treated with Cl_2, and 2 sets of seeds irradiated to doses of 0.25, 0.50, 0.75, 1.0, 1.25, or 1.50 kGy; 1 set of the irradiated seeds was further treated with 16000 ppm of Cl_2, as described below. The concentration of the Cl_2 solution was determined after appropriate dilution, as described above. The surviving *Salmonella* CFU in the control and γ-irradiated samples were enumerated, as described above.

The samples designated for Cl_2 were treated as follows: Inoculated irradiated or control seeds (10 g) were rinsed twice with 50 mL of sterile tap water with agitation for 2 min. After the final rinsate was removed, 50 mL of calcium hypochlorite (3% w/v), prepared in 500-mM potassium phosphate buffer, pH 6.8 (providing 16000 ppm of free chlorine) was added and mixed by hand massaging the pouch with the washed seeds for 10 min. The chlorine solution was removed and the treated seed rinsed twice with 50 mL of sterile tap water, as described above. After removal of the final rinsate, 20 mL of sterile 0.1% peptone water was added, and the mixture was pummeled for 1 min at normal speed. Serial decimal dilutions were prepared in sterile 0.1% peptone water, and dilutions were plated in triplicate to TSA (0.1 mL per plate). Plates were incubated at 37 °C, and colonies were counted at 24 and again after 48 h of incubation.

Statistical analysis

The data were analyzed by the GLM procedure, including an estimate of synergy, the Duncan-Waller multiple comparison test, and by analysis of covariance of the SAS statistical package (SAS 1999). Statistical significance was accepted at a level of $P < 0.05$.

Results and Discussion

Studies of antimicrobial treatments of *Salmonella* cells in vitro on membrane filters

The results of comparisons of controls to treatments of *Salmonella* cells in vitro on filters with 0.5 ppm Cl_2 solution for 10 min, γ-irradiation to a dose of 0.2 kGy, and γ-irradiation (0.2 kGy) followed by treatment with a solution of 0.5 ppm Cl_2 are presented in Table 1. A Cl_2 concentration of only 0.5 ppm was required to reduce the population of *Salmonella* cells on the membrane filter by 1.73 logs. The sum of the 2 independent treatments was a reduction in the population of *Salmonella* by 2.66 logs; since chlorination of the irradiated cells produced an inactivation of 3.46 logs, synergy occurred between the treatments. A test of synergy produced an estimate of 0.80 ± 0.16, a t value of −4.99, and a Pr > t of <0.0001.

Studies of antimicrobial treatments of *E. coli* O157:H7 in vitro on membrane filters

The results of comparisons of controls to treatments of *E. coli* O157:H7 in vitro on membrane filters with 0.5 ppm of Cl_2 solution for 10 min, γ-irradiation to a dose of 0.2 kGy, and γ-irradiation (0.2 kGy) followed by treatment with a solution of 0.5 ppm Cl_2 are

Table 1—Studies of antimicrobial treatments of *Salmonella* cells in vitro on membrane filters

Treatment	N[a]	Treated Log CFU ± SD	Log reduction ± SD
Control	9	6.70 ± 0.04	0.00 ± 0.04A
0.2 kGy γ-irradiated	9	5.76 ± 0.07	0.94 ± 0.06B
0.5 ppm Cl_2-treated	9	4.97 ± 0.39	1.73 ± 0.40C
γ-irradiated + Cl_2-treated	9	3.24 ± 0.26	3.46 ± 0.26D

[a]Means are of 3 independent experiments with 3 replicate samples per treatment, each with 3 Petri plates counted per replicate.
Means with the same letter are not significantly different by the Waller-Duncan k-ratio t test for the variable log reduction. k-ratio = 100 df = 32 MSE = 0.057069 F = 339.34; critical value of t = 1.80877. Minimum significant difference = 0.2037; the r^2 for the ANOVA = 0.970.

presented in Table 2. The sum of the 2 independent treatments was a reduction in the population of *E. coli* O157:H7 by 3.21 logs; since chlorination of the irradiated cells produced an inactivation of 3.93 logs, synergy occurred between the treatments. A test of synergy produced an estimate of 4.79 ± 0.10, a *t* value of 49.81, and a Pr > *t* of <0.0001.

Studies of antimicrobial treatments of *L. monocytogenes* cells in vitro on membrane filters

The results of comparisons of controls to treatments of *L. monocytogenes* cells in vitro on membrane filters with 0.5 ppm Cl_2 solution for 10 min, γ-irradiation to a dose of 0.2 kGy, and γ-irradiation (0.2 kGy) followed by treatment with a solution of 0.5 ppm Cl_2 are presented in Table 3. The sum of the 2 independent treatments was a reduction in the population of *L. monocytogenes* by 2.90 logs; since chlorination of the irradiated cells produced an inactivation of 3.48 logs, synergy occurred between the treatments. A test of synergy produced an estimate of 4.80 ± 0.07, a *t* value of 68.62, and a Pr > *t* of <0.0001.

Studies of antimicrobial treatments of *Salmonella* cells on 0.1 g of alfalfa seeds on membrane filters

The results of treating *Salmonella* on alfalfa seeds with 0.2 kGy of γ-radiation, 0.5 ppm Cl_2 for 10 min, or 0.2 kGy of γ-radiation followed by 0.5 ppm Cl_2 for 10 min, compared to untreated controls, are presented in Table 4. The inactivation of *Salmonella* by either γ-irradiation or chlorination was less when the pathogen was adsorbed onto alfalfa seed. The sum of the 2 independent treatments was a reduction in the population of the *Salmonella* by 1.48 logs. Since irradiation followed by chlorination produced a reduction of 2.31 logs, synergy occurred between the treatments. A test of synergy produced an estimate of −0.84 ± 0.15, a *t* value of −5.61, and a Pr > *t* of <0.0001.

Studies of antimicrobial treatments of *Salmonella* cells on 5.0 g of alfalfa seeds on membrane filters

The results of treating *Salmonella* on alfalfa seeds with 2.0 kGy of γ-radiation, 5000 ppm Cl_2 for 10 min, or 2.0 kGy of γ-radiation followed by 5000 ppm Cl_2 for 10 min, compared to untreated controls, are presented in Table 5. The inactivation of *Salmonella* by either γ-irradiation or chlorination was less when the pathogen was adsorbed onto alfalfa seed. The use of a D/E neutralizing broth wash greatly decreased the effectiveness of the treatment with Cl_2, requiring an increase in concentration from 0.5 ppm to 5000 ppm. The sum of the 2 independent treatments was a reduction in the population of the *Salmonella* by 2.11 logs. Since irradiation followed by chlorination produced a reduction of 2.21 logs, there was only a weak indication that synergy occurred between the treatments and the test of synergy using the GLM procedure was not significant: estimate of −0.10 ± 0.11, a *t* value of −0.91, and a Pr > *t* of <0.4153. Industrial practice does not involve a neutralization step to eliminate residual chlorine.

Determination of the *D*-value for the inactivation of *Salmonella* on 10 g seed by γ-irradiation and effectiveness of or by γ-irradiation followed by chlorination

The *D*-value for the γ-irradiated *Salmonella* on the alfalfa seeds was 1.20 ± 0.08 kGy, calculated from the experimental results summarized in Table 6. This value is higher than the authors previously reported (0.94 ± 0.03 and 1.02 ± 0.03 kGy) for 2 different lots of alfalfa seed inoculated with the same cocktail of *Salmonella* (Thayer and others 2003b). The γ+chlorination *D*-value for the γ-irradiated *Salmonella* when treated with 16000 ppm of Cl_2 was 0.84 ± 0.18 kGy. The regressions were determined to be significantly different (Pr > $F = 0.0403$) by analysis of covariance. If the effect of treating with chlorine were just additive, then the *D*-values for the γ-irradiated and the γ-irradiated followed by chlorination samples would be the same. In this experiment a dose of 1.5 kGy killed 1.72 ± 0.36 logs of *Salmonella*. Chlorination alone killed 3.58 ± 0.60 logs of cells. The

Table 2—Studies of antimicrobial treatments of *E. coli* O157:H7 cells in vitro on membrane filters

Treatment	N[a]	Treated log CFU ± SD	Log reduction ± SD
Control	3	6.64 ± 0.06	0.00 ± 0.06 A
0.2 kGy γ-irradiated	3	5.46 ± 0.06	1.18 ± 0.06 B
0.5 ppm Cl_2-treated	3	4.61 ± 0.03	2.03 ± 0.03 C
γ-irradiated + Cl_2-treated	3	2.71 ± 0.14	3.93 ± 0.14 D

[a]Means are of 3 independent experiments with 1 replicate per treatment, each with 3 Petri plates counted per replicate.
Means with the same letter are not significantly different by the Waller-Duncan *k*-ratio *t* test for the variable log reduction. *k*-ratio = 100 df = 8 MSE = 0.006901 $F = 1189.06$; critical value of $t = 2.13490$. Minimum significant difference = 0.1448; the r^2 for the ANOVA = 0.998.

Table 3—Studies of antimicrobial treatments of *L. monocytogenes* cells in vitro on membrane filters

Treatment	N[a]	Treated log CFU ± SD	Log reduction ± SD
Control	3	6.29 ± 0.02	0.00 ± 0.02A
0.2 kGy γ-irradiated	3	5.50 ± 0.02	0.79 ± 0.02B
0.5 ppm Cl_2-treated	3	4.17 ± 0.09	2.11 ± 0.09C
γ-irradiated + Cl_2-treated	3	2.81 ± 0.08	3.48 ± 0.08D

[a]Means are of 3 independent experiments with 1 replicate per treatment, each with 3 Petri plates counted per replicate.
Means with the same letter are not significantly different by the Waller-Duncan *k*-ratio *t* test for the variable log reduction. *k*-ratio = 100 df = 8 MSE = 0.003669 $F = 1908.38$; critical value of $t = 2.13434$. Minimum significant difference = 0.1056; the r^2 for the ANOVA = 0.999.

Table 4—Studies of antimicrobial treatments of *Salmonella* cells on 0.1 g of alfalfa seeds on membrane filters

Treatment	N[a]	Treated log CFU ± SD	Log reduction ± SD
Control	4	6.26 ± 0.08	0.00 ± 0.08A
0.2 kGy γ-irradiated	4	5.85 ± 0.11	0.41 ± 0.12B
0.5 ppm Cl_2-treated	4	5.19 ± 0.08	1.07 ± 0.08C
γ-irradiated + Cl_2-treated	4	3.95 ± 0.26	2.31 ± 0.25D

[a]Means are of 2 independent experiments each with 2 replicates for each treatment and 3 replicate Petri plates counted per replicate.
Means with the same letter are not significantly different by the Waller-Duncan *k*-ratio *t* test for the variable log reduction. *k*-ratio = 100, df = 12, MSE = 0.02231, $F = 182.25$; critical value of $t = 1.97991$. Minimum significant difference = 0.2091; the r^2 for the ANOVA = 0.978.

Table 5—Studies of antimicrobial treatments of *Salmonella* cells on 5.0 g of alfalfa seeds on membrane filters

Treatment	N[a]	Treated log CFU ± SD	Log reduction ± SD
Control	2	8.16 ± 0.00	0.00 ± 0.00A
2.0 kGy γ-irradiated	2	6.81 ± 0.16	1.36 ± 0.16C
5000 ppm Cl_2-treated	2	7.42 ± 0.02	0.75 ± 0.02B
γ-irradiated + Cl_2-treated	2	5.96 ± 0.03	2.21 ± 0.03D

[a]Means are of 2 independent experiments each with 1 replicate for each treatment and 3 replicate Petri plates counted per replicate.
Means with the same letter are not significantly different by the Waller-Duncan *k*-ratio *t* test for the variable log reduction. *k*-ratio = 100, df = 4, MSE = 0.006456, $F = 271.84$; critical value of $t = 2.84605$. Minimum significant difference = 0.2287; the r^2 for the ANOVA = 0.995.

Table 6—Inactivation of *Salmonella* on 20 g of alfalfa seeds by γ-irradiation or by γ-irradiation followed by chlorination

Treatment	kGy	0.00	0.25	0.50	0.75	1.00	1.25	1.50
γ	log CFU/g[a]	8.75 ± 0.10	8.16 ± 0.17	7.98 ± 0.11	7.68 ± 0.43	7.69 ± 0.16	7.42 ± 0.14	7.04 ± 0.36
	log kill	0	0.59 ± 0.17	0.78 ± 0.11	1.08 ± 0.43	1.07 ± 0.16	1.33 ± 0.14	1.72 ± 0.36
$\gamma + Cl_2$	log CFU/g	5.17 ± 0.70	4.74 ± 0.86	4.62 ± 0.96	4.49 ± 0.85	4.05 ± 0.84	4.04 ± 0.78	3.09 ± 1.56
	log kill	3.58 ± 0.60	4.01 ± 0.76	4.13 ± 0.86	4.26 ± 0.75	4.70 ± 0.74	4.71 ± 0.68	5.66 ± 1.46

[a]Logarithm of the mean of CFU/g of seeds from 6 independent experiments.

sum of the 2 treatments was 5.3 logs, which is less than the observed reduction of 5.66 logs for irradiation followed by chlorination.

Conclusion

Irradiation and chlorination acted synergistically, producing a greater-than-additive inactivation of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*. This synergistic interaction between the effects of γ-irradiation and chlorination was found to occur during the inactivation of *Salmonella* contaminating alfalfa seeds.

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